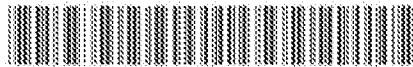




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Remarks

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) Protein disulfide isomerase gene derived from strain of methylotrophic yeast

(57) A protein derived from a strain of methylotrophic yeast which has a protein disulfide isomerase activity having the amino acid sequence as set forth in SEQ ID No. 1, or protein in which said amino acid sequence has

been modified by deletion or addition of one or a few amino acids, or substitution with other amino acid(s) and which has a protein disulfide isomerase activity; and a process for production thereof.

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## Description

The present invention relates to protein disulfide isomerase, an enzyme which promotes formation of protein conformation by catalyzing formation of disulfide bonds in a protein, and to a gene thereof. The present invention relates, among the protein disulfide isomerases, to protein disulfide isomerase derived from a strain of methylotrophic yeast, a microorganism suitable for industrial production of valuable proteins due to its high efficiency of expression of heterologous genes and secretion of the expression products, and to a gene thereof.

Protein disulfide isomerase (PDI) is a major protein present in the lumen of the endoplasmic reticulum (referred to hereinafter as ER) and it was first discovered as having an activity which effects oxidative refolding of a reduced RNase (Goldberger, R.F. et al. (1983) J. Biol. Chem. 258: 628-635). PDI is believed to be an enzyme which catalyzes formation of stable conformation by recombining disulfide bonds of secretory proteins.

It has been pointed out that, in the case of heterologous proteins, in particular secretory proteins, which often have disulfide bonds, recombination of disulfide bonds by PDI as well as protein folding by peptidyl-prolyl-cis-trans isomerase (PPI) represent the rate-limiting step in the secretory process of proteins (Gething, M.J. and Sambrook, J. (1992) Nature 355: 33-45). It has also been demonstrated that PDI promotes folding of proteins consisting of a single domain such as RNase *in vitro* as well (Jaenische, R. (1993) Curr. Opin. Struct. Biol. 3: 104-102).

On the other hand, because strain of methylotrophic yeasts grow using methanol as the sole carbon source and they have high yields of cells, they have been used for the production of materials for use in the synthetic chemical industry including, for example, aldehydes such as formaldehyde, epoxides, methylethyketone, and formic acid. Research has been conducted on the possible utilization of the cells *per se* as a protein source, and the utilization for production of cell components such as amino acids, vitamins, and the like, and some have been put into practical use. In recent years, furthermore, an expression system of heterologous genes using strain of methylotrophic yeasts as the host has been developed and it has been shown that said system has a higher productivity than Saccharomyces yeasts (Japanese Unexamined Patent Publication (Kokai) No. 5-344895).

Its productivity is high especially for secretory proteins. For example, the productivity of glucoamylase derived from filamentous fungi of the genus Rhizopus was 3.4 g/l, which is about 10 times higher than the productivity by Saccharomyces yeasts (Sakai, Y., et al. (1996) Biochim. Biophys. Acta 1208: 81-87). As the strain of methylotrophic yeast, there are known Candida boidinii, Pichia pastoris, Hansenula polymorpha, and the like.

When heterologous proteins are produced by secretory production using recombinant DNA technology, the efficiency of the secretion is thought to be increased by enhancing the speed of folding proteins. Based on such an idea, an example has been disclosed in which the amount secreted of human albumin was increased by about 60% on the average by coexpressing a human PDI gene with the desired gene in a Saccharomyces yeast (Japanese Unexamined Patent Publication (Kokai) No. 5-38771).

Formation or exchange of disulfide bonds which are necessary for appropriate folding of proteins requires environments suitable therefor. For that purpose, eukaryotic cells have intracellular compartments such as the ER or the Golgi apparatus, etc. While passing through the compartments, secretory proteins are subjected to suitable folding or addition of sugar chains and then are secreted out of the cell by means of exocytosis. Many of the secretory proteins of eukaryotic origin have intramolecular disulfide bonds, and formation and exchange of these disulfide bonds taking place in the ER are essential for formation of protein conformation and its secretion.

Accordingly, the PDI which catalyzes reactions for formation and/or exchange of disulfide bonds must be localized or stay in the ER. For this purpose the PDI has a unique amino acid sequence called an ER retention signal sequence at the C-terminal. As ER retention signal sequences there are known Lys-Asp-Glu-Leu (SEQ ID No. 2) for animals and His-Asp-Glu-Leu (SEQ ID No. 3) for Saccharomyces yeasts. When the human PDI gene as described above was expressed in a Saccharomyces yeast, the ER retention signal sequence of the human PDI did not fully function, which was possibly due to inadequate localization of the PDI in the ER. Thus, it is believed that even the highly expressed PDI gene did not cause enhancement in the PDI activity commensurate with the expression in the ER, and accordingly the increment of the amount secreted of the coexpressed secretory protein remained at a value of 60%.

In order for the PDI expressed in a strain of methylotrophic yeast to fully perform its functions, it is preferred to use the PDI derived from a strain of methylotrophic yeast. The reason why the strain of methylotrophic yeast has a high ability of secreting protein as described above is that recombination of disulfide bonds by the PDI, which is the rate-limiting step of the protein secretion process takes place efficiently and that the PDI derived from the strain of methylotrophic yeast has a higher specific activity than the PDI derived from other sources or has a higher activity in the ER. However, the PDI of the strain of methylotrophic yeast or the gene thereof was unknown. Accordingly, no studies had been carried out on enhancement of productivity in the expression system of the strain of methylotrophic yeast by using the above PDI or the gene thereof.

The inventors have carried out intensive studies to clone the PDI gene carried by strain of methylotrophic yeast, to elucidate the nucleotide sequence thereof, and to reveal the characteristics of the PDI of strain of methylotrophic yeast. Thus, it is the object of the present invention to provide the PDI gene derived from strain of methylotrophic yeast

in order to effect secretory production of heterologous genes by strain of methylotrophic yeast in a more efficient manner.

In order to attain the above-mentioned objective, the inventors have obtained a DNA fragment amplified by the PCR using as a primer an oligonucleotide synthesized based on an amino acid sequence of the conserved region present in the active site of the PDI. By means of the colony hybridization method using this amplified DNA fragment as a probe, the inventors have cloned the PDI gene of the strain of methylotrophic yeast Candida boidinii, and demonstrated the nucleotide sequence of said gene and the amino acid sequence of said PDI. Furthermore, by coexpressing the peroxidase gene derived from a filamentous fungus in the strain of methylotrophic yeast transformed with said PDI gene, the inventors have successfully increased by about 10 times the amount secreted of said peroxidase and have accomplished the present invention.

Thus, the present invention provides a protein derived from a strain of methylotrophic yeast which has a protein disulfide isomerase activity having the amino acid sequence as set forth in SEQ ID No. 1, or protein in which said amino acid sequence has been modified by deletion or addition of one or a few amino acids, or substitution with other amino acid(s) and which has a protein disulfide isomerase activity. The present invention also provides a gene encoding the PDI, a vector comprising said gene, and a host transformed with said vector, as well as a process for secreting in large amounts the desired protein by coexpressing the gene for said desired protein in said transformed yeast host.

#### BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 is a drawing that shows a restriction enzyme map of the 6.2 kb DNA fragment containing the PDI1 gene of Candida boidinii, the region for which the nucleotide sequence was determined, and the position and direction of the PDI1 gene thereof.

Fig. 2 is a drawing that shows the result of Southern hybridization demonstrating the presence of the PDI gene in C. boidinii.

Fig. 3 (a) is a drawing that shows the construction of the expression vector pNPO2 of the ARP gene and (b) is a drawing that shows the construction of the expression vector pNRPD of the PDI1 gene.

Fig. 4 is a drawing that shows a procedure of construction of the expression vector pNRPD for the PDI1 gene.

Fig. 5 is a schematic diagram showing the state in which the ARP gene has been integrated into the genomic DNA of the BPO17 strain, and a drawing that shows the result of Southern hybridization confirming it.

Fig. 6 is a schematic diagram showing the state in which the PDI1 gene has been integrated into the genomic DNA of the BPP1 strain, and a drawing that shows the result of Southern hybridization confirming it.

Fig. 7 is a drawing that shows the result of Northern analysis which analyzed the amount of the expressed PDI1 gene.

Fig. 8 is a drawing that shows the ARP activity in the culture liquid of each of the cultured BPO17 strain, the BPP1 strain, and the BUL strain.

#### DETAILED DESCRIPTION

The present invention is now explained in detail below.

First, the sequence, Cys-Gly-His-Cys, which is conserved in PDI's from a variety of sources as the active center of the exchange reaction of disulfide bonds was found at two sites in the amino acid sequence of the PDI derived from Saccharomyces cerevisiae. Based on the amino acid sequence of PDI of S. cerevisiae comprising said sequence, various primers for the PCR were designed with reference to the frequency of use of codons from the strain of methylotrophic yeast. Using these primers PCR reactions were carried out using the genomic DNA of the strain of methylotrophic yeast as a template, and the amino acid sequence deduced from the nucleotide sequence of the PCR reaction product thus obtained was confirmed to be analogous to the amino acid sequence of PDI of S. cerevisiae.

The genomic DNA of a strain of methylotrophic yeast is completely digested with various restriction enzymes and is fractionated on agarose gel electrophoresis. Using the above-mentioned PCR products as a probe, Southern hybridization is carried out to find a restriction enzyme which gives the smallest DNA fragment containing the entire region of the PDI gene. Using the genomic DNA of the strain of methylotrophic yeast which has been completely digested with the restriction enzyme, a genomic library is created, which is then subjected to colony hybridization using the above-mentioned PCR product as a probe to select clones having the PDI gene.

Plasmid is extracted from the selected clone, and is subjected to Southern hybridization to confirm that the plasmid contains the sequence of the above-mentioned PCR product. Furthermore, a restriction map of the inserted fragments of this plasmid is created, based on which subcloning is conducted to obtain the smallest DNA fragment containing the PDI gene. The nucleotide sequence of the DNA fragment obtained is determined and the amino acid sequence of the PDI derived from the strain of methylotrophic yeast is analyzed.

The PDI gene thus obtained derived from the strain of methylotrophic yeast can be highly expressed in the strain of methylotrophic yeast to prepare the PDI. As an expression vector for the PDI gene known vectors may be used,

and as an expression vector for the strain of methylotrophic yeast Candida boidinii, pNOTe1 or pTRex as described in Japanese Unexamined Patent Publication (Kokai) No. 5-344896 may be used. As the method for transforming the strain of methylotrophic yeast and the method for obtaining a transformant in which a foreign gene has been integrated into the chromosomal DNA thereof, a known method (Sakai, Y. et al. (1991), J. Bacteriol. 173: 7458-7463) can be used. Furthermore, the amount secreted of the desired secretory protein can be enhanced by coexpressing the PDI gene derived from the strain of methylotrophic yeast with the gene of the desired secretory protein in the strain of methylotrophic yeast.

Although the PDI derived from the strain of methylotrophic yeast had the ER retention signal Arg-Asp-Glu-Leu (SEQ ID No. 9) which is different from His-Asp-Glu-Leu (SEQ ID No. 8) derived from a Saccharomyces yeast, there is no doubt that the cells of C. boidinii recognize the former sequence which is of its own and the PDI is retained by the ER to fully perform its function. As expression vectors employed for expression of the PDI, those in which auxotrophic markers such as the above-mentioned pNOTe1 and pTRex have been replaced with the genes different from the ones used for the expression vector of the desired protein may be used. Furthermore, by imparting to the host strain of methylotrophic yeast auxotrophy corresponding to the two markers of the expression vector, transformation is possible by the method as described above. As the method for imparting auxotrophy to the strain of methylotrophic yeast, a known method (Sakai, Y. et al. (1991), J. Bacteriol. 173: 7458-7463) can be used.

#### EXAMPLES

The invention will be understood more readily with reference to the following examples; however these examples are intended to illustrate the invention and are not to be construed to limit the scope of the invention.

##### Example 1:

From Candida boidinii strain S2 (Tani, Y. et al. (1985) Agric. Biol. Chem. 49: 2699-2708), the PDI gene was obtained and the nucleotide sequence thereof was determined. Incidentally, said strain has been designated Candida boidinii SAM1968 and deposited as an international deposition under the Budapest Treaty on February 25, 1992, with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, MITI, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, 305, Japan, with the accession number FERM BP-3768.

##### (1) Amplification by PCR

Two sequences were noted as the amino acid sequences in the PDI of S. cerevisiae relating to Cys-Gly-His-Cys (SEQ ID No. 4), a sequence which is conserved in the PDI of various origins as the active center of the disulfide bond exchange reaction:

Pro-Trp-Cys-Gly-His-Cys-Lys (SEQ ID No. 5)

(amino acids No. 63 through No. 65 in the amino acid sequence of the PDI of Saccharomyces yeast).

Tyr-Ala-Pro-Trp-Cys-Gly-His (SEQ ID No. 6)

(amino acids No. 402 through No. 408 in the amino acid sequence of the PDI of Saccharomyces yeast).

Referring to the frequency of use of C. boidinii codons, oligonucleotides having the following nucleotide sequences corresponding to the amino acid sequence was synthesized:

That is, as the sense primer,

5' -CCCGGAATTC CCT(A) TCG TGT(C) GGT(A) CAT(C) TGT(C) AA-3'  
(SEQ ID NO. 7),

and as the antisense primer,

5'-GGGGATCC TG A(T)CC A(G)CA CCA A(T)CC A(G/T)GC  
A(G)T-3' (SEQ. ID NO. 8)

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were synthesized. These oligonucleotides have on their 5'-end the sequence which recognizes EcoRI and BamHI, respectively. They are so designed that an EcoRI site is formed at the 5'-end and a BamHI site is formed at the 3'-end of the DNA fragments amplified by these two primers.

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When PCR reaction was carried out using the genomic DNA of C. boidinii as a template and the above two oligonucleotides as a primer, an amplified DNA fragment of about 1 kb was observed. The amplified fragment was recovered and a DNA fragment of about 250 bp obtained by digestion of said fragment with a restriction enzyme EcoRI was inserted into the EcoRI-digested pBluescript II SK+. Analysis of the nucleotide sequence of the inserted fragment revealed a nucleotide sequence encoding an amino acid sequence having a high homology with the amino acid sequence of the PDI of S. cerevisiae, and therefore this DNA fragment was concluded to be part of the PDI gene of C. boidinii.

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#### (2) Southern hybridization analysis of the genomic DNA

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Genomic DNA was isolated from the bacterial cells of Candida boidinii strain S2. As the method for isolating DNA, there is mentioned a method by Cryer (Cryer, D.R. et al. (1975) Meth. Cell. Biol. 12: 39-44). The genomic DNA of Candida boidinii strain S2 was cleaved with various restriction enzymes and then separated on a 0.7% agarose gel by electrophoresis. The separated DNA was transferred to and immobilized on a nylon membrane (manufactured by Amersham). A 250 bp DNA fragment containing the above-mentioned PDI gene was labelled with 32P using the Random Primer kit (manufactured by Amersham).

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The labelled DNA fragment was added to a 5 x SSC - 1% SDS - 1 x Denhardt solution to prepare a hybridization solution. This hybridization solution was added to the DNA-immobilized nylon membrane and encapsulated in a plastic bag. After the encapsulated plastic bag was incubated at 65°C for 16 hours, the nylon membrane was removed from the plastic bag and washed in a 2 x SSC - 0.1% SDS solution at room temperature. Subsequently the nylon membrane was incubated in a 0.2 x SSC - 0.1% SDS solution and after the solution was replaced with a new one incubation at 65°C for 30 minutes was repeated. After the membrane was washed in a 2 x SSC, it was air-dried and subjected to autoradiography. As the smallest DNA fragment hybridizing to the above-mentioned 250 bp probe, an XbaI fragment of about 6.2 kb was found as shown in Fig. 2.

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#### (3) Cloning of the PDI gene by colony hybridization

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The genomic DNA of Candida boidinii strain S2 was completely digested with a restriction enzyme XbaI and fractionated on a 0.7% agarose gel electrophoresis. The agarose at around 6.2 kb was excised and the DNA fragment was recovered using a DNA cell (manufactured by Daichi Kagaku). The recovered DNA was inserted into the XbaI-digested pBluescript II SK+, and Escherichia coli strain JM109 was transformed to prepare the genomic library of Candida boidinii strain S2.

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The library was screened by colony hybridization using the above-mentioned 250 bp DNA fragment as a probe to obtain positive clones. The hybridization conditions were the same as that of the above-mentioned Southern hybridization. Plasmid was recovered from the positive clones to create a restriction enzyme map of the inserted DNA fragments. The restriction enzyme map so created is shown in Fig. 1. Subcloning was carried out based on the restriction enzyme map and the DNA fragment containing the PDI gene was limited to about 2 kb (the left hand side in Fig. 1) spanning from XbaI to SalI.

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#### (4) Determination of the nucleotide sequence

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The nucleotide sequence of the above DNA fragment of about 2 kb spanning from XbaI to SalI was determined. The DNA fragment was cloned into phage M13 in the both directions to prepare each of the double stranded DNA's (DS). These double stranded DNA's were allowed to react with Escherichia coli exonuclease III to prepare a double stranded DNA in which deletion has been introduced in one direction. A method for making an plasmid having a one-direction deletion insertion using exonuclease III has been described in detail on pages 289-305 in "Zoku Seikagaku Jikken Kouza (Sequel to the Series of Biochemistry Experiments), Vol. 1, Idenshi Kenkyuuhou (Methods for Studying Genes) II".

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Each of the double stranded DNA's in which deletion has been inserted in one direction obtained in the above method was transformed into E. coli strain JM109 to make a phage clone in which deletion has been inserted in one

direction. From each phage clone a double stranded DNA was prepared, for which the degree of deletion was investigated from the cleavage pattern by restriction enzymes, and then single stranded phage DNA's were prepared from appropriate clones. Using these single stranded phage DNA's as the template, the nucleotide sequence was determined by the dideoxy method (Sanger, F. et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74: 5463). By ligating the nucleotide sequence of each clone the nucleotide sequence of 2.0 kb spanning from the *Xba*I site to immediately before the *Sall* site in Fig. 2 was determined.

SEQ ID No. 1 shows the nucleotide sequence and the amino acid sequence of the PDI deduced from the nucleotide sequence. The PDI of *C. boidinii* was found to consist of 531 amino acids encoded by the nucleotide sequence from the base No. 367 through 1959 of the nucleotide sequence shown in SEQ ID No. 1, and was designated the PDI1 gene. The amino acid sequence of the PDI1 has shown a homology of 45% with the PDI derived from *S. cerevisiae* and 22% with the human PDI. When analogous amino acids are considered, the homology was 64% with the PDI of *S. cerevisiae* and 49% with the human PDI.

The sequence which has been conserved in the PDI of various origins as the active center of the disulfide bond exchange reaction of the PDI, i.e., Cys-Gly-His-Cys (SEQ ID No. 4), was found in two sites: the amino acid sequence from amino acids 61 to 64 and that from amino acids 408 to 411 of the amino acid sequence of SEQ ID No. 1. Furthermore, the ER retention signal sequence present in the C-terminal was Arg-Asp-Glu-Leu (SEQ ID No. 9), which was different from the PDI of *S. cerevisiae*, His-Asp-Glu-Leu (SEQ ID No. 3), or Lys-Asp-Glu-Leu (SEQ ID No. 2) widely occurring in the PDI of mammals.

The measurement of the activity of protein disulfide isomerase can be performed by investigating the accelerating effect on reassembly of the scrambled ribonuclease A (RNase A) which was made by a method comprising reduction, denaturation and reoxidation. The degree of reassembly of ribonuclease A is quantitated using the degree of recovery of the enzymatic activity as an index (Japanese Unexamined Patent Publication (Kokai) No. 6-38771).

By measuring the PDI activity by the above-mentioned method, it was confirmed that the strain of methylotrophic yeast transformant containing the above DNA fragment had a higher protein disulfide isomerase activity than the untransformed strain of methylotrophic yeast as the control as shown in Fig. 3.

#### Example 2. Secretion of the desired heterologous protein

It was confirmed that the amount secreted of APP is increased by coexpressing the PDI1 gene derived from the strain of methylotrophic yeast *C. boidinii* and the peroxidase gene (APP) gene derived from a filamentous fungus *Arthromyces ramosus*. pNOTe1 used as an expression vector and the APP expression vector pNOTe1APP have been disclosed in Japanese Unexamined Patent Publication (Kokai) No. 5-344895. By exchanging the auxotrophic marker (URA3) of pNOTe1 for the LEU2 gene derived from *C. boidinii*, an expression vector having an auxotrophic marker different from pNOTe1 can be created. It is also possible to effect transformation by the two expression vectors mentioned above, by imparting to the strain of methylotrophic yeast auxotrophy corresponding to the markers of these two expression vectors. As a method for imparting auxotrophy to the strain of methylotrophic yeast, a known method (Sakai, Y. et al. (1991) J. Bacteriol. 173: 7458-7463) can be used.

##### (1) Construction of expression vectors

A 1.1 kb EcoRI DNA fragment containing the APP gene was excised from plasmid pNOTe1APP, and then inserted into the *Nos*I site of pNCTe1 to create plasmid pNPO3 as shown in Fig. 3 (a).

For the purpose of expressing the PDI1 gene, an expression vector having the LEU2 gene as an auxotrophic marker and the ribosome DNA (rDNA) of *C. boidinii* as a recombination site was created in the procedure as set forth in Fig. 4. To begin with, pNOTe1 was cleaved with EcoRI and HindIII and then a 2.0 kb DNA fragment containing the promoter and terminator of the alcohol oxidase gene (AOX1) of *C. boidinii* was excised and inserted into the EcoRI-HindIII site of pUC19 to create plasmid pNCT46. A DNA fragment containing rDNA derived from *C. boidinii* was obtained by the PCR method and was then inserted into the HindIII site of pNOT46 to create pNOT46R. Plasmid pCLEU321 (Sakai, Y. et al. (1992) J. Bacteriol. 174: 5989-5993) containing the LEU2 gene of *C. boidinii* was digested with EcoRI, and a DNA fragment containing a 3.2 kb LEU2 gene, which was rendered blunt-ended. After the blunt-ended 3.2 kb DNA fragment was digested with NdeI, it was inserted into the blunt-ended pNOT46R to create pNL1.

In order to integrate the above expression vectors, a *Nos*I site was created on both ends of the PDI gene by the PCR method. As the sense primer,

5' -ATAAGAATGGGGCGAAATGAACCTAACTAATTTCAAA-3' (SEQ ID No. 10);

and as the antisense primer.

5' -ATAAGAATGCCGGCCGCTTATAATTCAACCGAACATCA-3' (SEQ ID NO.  
8  
11)

were synthesized. At the 5'-end of these two oligonucleotides there is a sequence recognized by NcoI so that a NcoI site may be created immediately before the initiation codon and immediately after the termination codon of the PDI1 gene in a DNA fragment amplified using these primers. Using the genomic DNA of C. boldinii as a template and the above two primers as a primer, PCR reaction was carried out, and the amplified 1.6 kb DNA fragment was digested with NcoI, which was inserted into the NcoI site of plasmid pBluescript II SK+ to create pSKPD. A 1.6 kb DNA fragment obtained by digesting pSKPD with NcoI was inserted into the NcoI site of the above pNL1 to create pNRPD as shown in Fig. 3 (b).

15 (2) Creation of a transformed yeast

Using the two expression vectors mentioned above in (1), a transformant of the strain of methylotrophic yeast C. boldinii was created. The bacterial strain used as the host is C. boldinii BUL (ura3, leu2) wherein the LEU2 gene of C. boldinii strain TK62 (ura3) (disclosed in Japanese Unexamined Patent Publication (Kokai) No. 5-344895) has been destracted. The LEU2 gene of C. boldinii has been disclosed by Sakai et al. (Sakai, Y. et al. (1992) J. Bacteriol. 174: 5998-5999). The method of transformation of C. boldinii has been disclosed in Japanese Unexamined Patent Publication (Kokai) No. 5-344895.

To begin with, a transformant of the ARP gene was created. After the ARP expression vector pNPO3 was linearized by digestion with BarnHI, C. boldinii strain BUL (ura3, leu2) was transformed, and transformants were selected using Ura3+. As shown in Fig. 5 A, B), in one of the transformants selected, the BPO17 strain, the entire region of pNPO3 containing the ARP gene has been integrated into the ura3 site by homologous recombination of the ura3 site on the chromosomal DNA of the host yeast BUL strain and the URA3 site in the expression vector pNPO3. This was confirmed by the fact, as shown in Fig. 5 C), that in Southern hybridization carried out using as a probe a 3.3 kb BarnHI-Sall DNA fragment containing the entire region of the URA3 gene after the genomic DNA of the host BUL strain and the transformant BPO17 strain were digested with Sall, a 5.5 kb hybridizing band in the BUL strain and a 14.4 kb hybridizing band in the BPO17 strain were observed.

Next, using as the host C. boldinii strain BPO17 (leu2) which was transformed with the above-mentioned ARP gene, a transformant of the PDI1 gene was created. After the PDI1 expression vector pNRPD was linearized by digestion with Apal, the BPO17 (leu2) strain was transformed and the BPP1 strain was obtained after selection with Leu+. In the BPP1 strain, as shown in Fig. 6, A, B), the entire region of pNRPD containing the PDI1 gene has been integrated into the rDNA site by homologous recombination of the rDNA site on the chromosomal DNA of the host yeast BPO17 strain and the rRNA site in the expression vector pNPO3. Integration of the PDI1 gene into the chromosomal DNA was confirmed by the fact, as shown in Fig. 6 C), that in Southern hybridization carried out using as a probe a 1.6 kb DNA fragment containing the PDI1 gene obtained by digestion of pSKPD with NcoI after the genomic DNA of the BUL strain, the host BPO17 strain and the transformant BPP1 strain were digested with HindIII, a band derived from the region containing a 12.6 kb intrinsic PDI1 gene from the BUL strain and the BPO17 strain and a 6.1 kb band derived from the expression vector pNRPD in addition to the above 12.6 kb band from the BPP1 strain were observed.

46 (3) Analysis of transformants

mRNA was extracted from the BPO17 strain transformed with the ARP gene, the BPP1 strain transformed with the ARP gene and the PDI1 gene, and the BUL strain used as the host, and the amount expressed of the PDI1 gene was investigated by Northern hybridization. From the bacterial cells obtained from the above three strains cultured at 30°C for 48 hours in the YM medium having methanol as the sole carbon source (Sakai, Y. et al. (1981) J. Gen. Microbiol. 123: 365-396), total RNA was extracted by ISCOGEN (manufactured by Nihon Gene K.K.) and purified using BIOMAG mRNA purification kit (manufactured by PerSeptive Diagnostics). The purified mRNA was subjected to a 1.1% agarose gel electrophoresis (containing 20 mM MOPS buffer, 1 mM EDTA, 2.2 M formamide), and then blotted onto the nylon membrane. In the same condition as the Southern hybridization described in Example 1, hybridization was carried out. The probe used in the hybridization was 1.6 kb NcoI DNA fragment derived from the above-mentioned pSKPD.

As shown in Fig. 7, strong expression of the PDI1 gene was observed in the BPP1 strain which was transformed with the PDI1 gene and weak expression of possibly the intrinsic PDI1 gene was observed in the BUL strain and the

BPO17 strain.

The above three bacterial strains were cultured in the YM medium containing methanol as the sole carbon source at 30°C for 48 hours, and then the PDI activity in the bacterial cells was measured. The harvested cells were suspended in 50 mM potassium phosphate buffer, pH 7.5, and transferred into a 2 ml Eppendorf tube, to which was added an equal volume of zirconium beads (0.5 mm in diameter). A procedure of vigorous stirring of the tube for 30 seconds using the Beads Beader (Model 3110BX, Biospec Products) followed by cooling on ice for 30 seconds was repeated for six times. The disrupted cells were centrifuged at 4°C at 16,000 × g for 5 minutes and then the enzymatic activity of the supernatant was measured.

The measurement of the PDI activity was carried out in accordance with the method of Hisilson et al. (Hisilson, D. A. et al. (1984) Methods Enzymol. 107: 281-294). Thus, one ml of the final reaction mixture contains 50 mM potassium phosphate buffer, pH 7.5, 500 µg of scrambled RNase, and 0.01 mM of dithiothreitol. After the reaction mixture was incubated for 10 minutes, 10 µl was sampled out, to which 3 ml of the TKM buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl<sub>2</sub>) containing 0.25 mg yeast RNA was added and then RNase activity was determined by measuring absorbance at 260 nm in a UV cuvette at 30°C for 2 minutes. One unit of the enzymatic activity was defined as the amount of enzyme which increases the absorbance at 260 nm per one minute.

As shown in Table 1, the PDI activity in the bacterial cells was higher in the BPP1 strain transformed with the PDI gene than the BPO17 strain transformed with the ARP gene alone or the BUL strain used as the host by a factor of 9 or more.

Table 1

Strain Enzyme	BUL	BPO17	BPP1
PDI	<0.1*	<0.1*	0.896

\* The levels of BUL and BPO17 were below the detection

limit.

#### (4) Secretory expression of the ARP

The BPO17 strain transformed with the ARP gene, the BPP1 strain transformed with both of the ARP gene and the PDI1 gene, and the BUL strain used as the host were cultivated in the YM medium containing methanol as the sole carbon source, and the ARP activity in the culture liquid was compared. As shown in Fig. 8, the ARP activity in the culture liquid of the BPP1 strain coexpressed with the ARP gene and the PDI1 gene reached a maximum of 0.024 U/ml at 84 hours after cultivation. In the BPO17 strain in which the ARP gene only was expressed, the ARP activity in the culture liquid reached a maximum of 0.002 U/ml at 84 hours after cultivation, while no ARP activity was observed in the culture liquid of the BUL strain used as the host. The result revealed that by coexpressing the PDI1 gene and the ARP gene the amount secreted of ARP increased by about 10 fold.

The present invention made it possible to obtain the PDI gene of the strain of methylotrophic yeast and to obtain the PDI enzyme by expressing said gene in large quantities using said strain of methylotrophic yeast. Furthermore, by coexpressing said gene with the gene of the desired secretory protein in the strain of methylotrophic yeast it became possible to drastically increase the amount produced of the desired protein.

Note: this invention extends to mutants or modified forms of the nucleotide sequence and protein sequence of SEQ ID 1, provided that these are associated with the protein disulfide isomerase activity, which at the least includes the ability to catalyse formation/exchange of protein disulfide bonds and location in the ER.

Likewise, the use of vectors containing such mutants (modified sequences to transform hosts so as to increase their PDI activity in particular for expressing a recombinant (heterologous) protein).

## SEQUENCE LISTING

SEQ ID No: 1

Sequence length: 2030

Sequence type: nucleic acid

Strandedness: double

Topology: linear

Molecule type: genomic DNA

Hypothetical: No

Antisense: No

Original source:

Organism: *Candida bovidinii*

Strain: S2

Sequence description:

AGAGCGCTCT	CCACTCACTC	ATTATTCATC	CAGTATCTCC	TCCAAGCTTG	TGAACAATT	60
CACTCACTTG	CCTTGCTTTA	CCATCTACTCA	ATCGTTCA	TTTACTCTG	TATCATTCGA	120
CCATTTCATC	ACTTTTCACT	ATCTACTAAT	AAATGTCTA	ACCAACGATA	ATCTTCAGC	180
AGATTCCGCTC	TTCCTTGATT	CAATTGATCT	TTCATAGAC	AGATCACTGA	CACCTGATA	240
CTTACATAGA	TATATATATA	TATATATGAA	ATTTACTTT	CCTCATTACT	CAATTGATTG	300
CATTAAATAC	ATTCATAGTA	TAATATATTGA	CITAAATAT	ATTTACATAT	ACACATAACA	360
TTTAAA	ATG AAG TTA ACT	AAT TTC AAA GTT ATT	GCC ACA ATT CTT GCT			408
Met Lys Leu Thr Asn Phe Lys Val Ile Ala Thr Ile Leu Ala						
1	5	10				
TGT TTA ACA GTT GTT AGA GCT GAT GAT GGT GGT GCG ATT GCA TCT CCA						456
Cys Leu Thr Val Val Arg Ala Asp Asp Gly Gly Ala Ile Ala Ser Pro						
15	20	25	30			
GAT TCC GCT GTT GTT AAA TTA ACT GCT GAT TCA TTC GAA TCA TTC ATG						504
Asp Ser Ala Val Val Lys Leu Thr Ala Asp Ser Phe Glu Ser Phe Met						
35	40	45				
AAA GAA AAT CCA TTA GTC TTA GCT GAA TTT TTT GCT CCT TGG TGT GGT						552
Lys Glu Asn Pro Leu Val Leu Ala Glu Phe Phe Ala Pro Trp Cys Gly						
50	55	60				
CAT TGT AAA AGA TTG GGT CCT GAA TTT CAA GTT GCT GCT GAT AAA TTA						600
Nis Cys Lys Arg Leu Glu Phe Glu Phe Cln Val Ala Ala Asp Lys Leu						
65	70	75				

	GTT GAA AAA GAT ATT AGA TTA CCT CAA ATT GAT TGT ACC GAA GAA AAA	648		
	Val Glu Lys Asp Ile Arg Leu Ala Glu Ile Asp Cys Thr Glu Glu Lys			
8	80	85	90	
	GAT TTA TGT TCT TAT GGT ATT AAA CCT TAG CGA ACT TTA AAA GTC	696		
	Asp Leu Cys Ser Ser Tyr Gly Ile Lys Gly Tyr Pro Thr Leu Lys Val			
16	95	100	105	110
	TTT AGA CCT TAC GAA AAT GAA CCT TCT GAT TAT CCT GGT CAA AGA ACT	744		
	Phe Arg Gly Tyr Glu Asn Gln Pro Ser Asp Tyr Ala Gly Gln Arg Thr			
25	115	120	125	
	TCA GAT TCA ATC ATT TCT TAT ATG CTT AAA CAA TCA ACC CGA CCT GTC	792		
	Ser Asp Ser Ile Ile Ser Tyr Met Val Lys Glu Ser Thr Pro Pro Val			
	130	135	140	
33	TCC ATC GTT GAT GAT CTC TCA GAT ATC GAA GAT AGA ATT AAA GAA TCA	840		
	Ser Ile Val Asp Asp Leu Ser Asp Ile Glu Asp Thr Ile Lys Glu Ser			
	145	150	155	
42	AAT GAT CCT GTC TTT ATT GAA GTC TTA CCA AAA GGT TCT AAA TCT GTC	888		
	Asn Asp Pro Val Phe Ile Gln Val Leu Pro Lys Gly Ser Lys Ser Val			
	160	165	170	
50	GAA GCC CGT AAC TCA ACT TTG TTT GAA ATC GCT AAT GGT TTA AGA GAT	936		
	Glu Ala Gly Asn Ser Thr Phe Phe Glu Ile Ala Asn Gly Leu Arg Asp			
	175	180	185	190
58	AAC TAC TCT TTT ATT TCA AGA ACA AGT ACT GAA TTC TCT TCA AAA TAC	984		
	Asn Tyr Ser Phe Ile Ser Thr Thr Ser Thr Glu Phe Ser Ser Lys Tyr			
	195	200	205	
66	TTG AAA GGT ATT AAA AAA TCA GAT ACT CCA CCT TAT ATT CTC TTT AGA	1032		
	Leu Lys Gly Ile Lys Lys Ser Asp Thr Pro Ser Tyr Ile Leu Phe Arg			
	210	215	220	
75	CCA AAT GAA GAA TTG CCT GAT CCT TCA ATC TAT AAA TTT GAT GAA ATT	1080		
	Pro Asn Glu Gln Leu Ser Asp Ala Ser Ile Tyr Lys Phe Asp Glu Ile			
	225	230	235	
83	GAT GAT ACT CAT TTA ATC GAA TTC TTA AAC CCT GAA TCA AAA CCT TTA	1128		
	Asp Asp Thr His Leu Ile Glu Phe Leu Asn Val Glu Ser Lys Pro Leu			
	240	245	250	
91	TTC CCT GAA ATG GAT GGT TCT TCT TTC GAA CCT TAT ATG CAA ATC AAA	1176		
	Phe Gly Glu Met Asp Gly Ser Ser Phe Gln Ser Tyr Met Glu Met Lys			
	255	260	265	270

EP 0 828 004 A2

	TTA CCA GTT GCT TAT TAT TTC TAT AAT GAA ATC TCT GAA AAA GAT GCC	1224		
5	Leu Pro Val Ala Tyr Tyr Phe Tyr Asn Glu Ile Ser Glu Lys Asp Ala			
	275	280	285	
10	GTC TCT GAT GCC ATC AGT AAA TTA GCT AAA ACT CAT AGA GGT AAA GTT	1272		
	Val Ser Asp Ala Ile Ser Lys Leu Ala Lys Thr His Arg Gly Lys Val			
	280	285	300	
15	AAT TTC GTT GGT TTA GAC CCT TCT AAA TAT GGT TTA CAC CCT AAG AAT	1320		
	Asn Phe Val Gly Leu Asp Ala Ser Lys Tyr Gly Leu His Ala Lys Asn			
	305	310	315	
20	ATT AAG ATG AAC GAA GAA TTC CCT CTT TTC GGT ATT CAC GAT TTA GCA	1368		
	Ile Asn Met Lys Glu Glu Phe Pro Leu Phe Ala Ile His Asp Leu Ala			
	320	325	330	
25	ACT GAA TTA AAA TAG GGT ATC TCC CAA GAT AAA CCA TTA GAT AAT AAA	1416		
	Thr Glu Leu Lys Tyr Gly Ile Ser Glu Asp Lys Pro Leu Asp Asn Lys			
	335	340	345	350
30	TTA ATT CCA AAA TTC GTT GAA GAT TTC GTT GGT GCT AAA TTA GAA GCA	1464		
	Leu Ile Pro Lys Phe Val Glu Asp Phe Val Ala Gly Lys Leu Glu Ala			
	355	360	365	370
35	ATG ATT AAA TCA GAA CCA ATC CCA GAA ACT CAA GAT TCT CCA GTT TAC	1512		
	Ile Ile Lys Ser Glu Pro Ile Pro Glu Thr Glu Asp Ser Pro Val Tyr			
	370	375	380	385
40	CAT TTA GTC GGT AAA GAA CAT GAT AAA ATT ATT ACC TCT GAT AAA GAT	1560		
	His Leu Val Gly Lys Glu His Asp Lys Ile Ile Thr Ser Asp Lys Asp			
	385	390	395	400
45	GTC TTA GTT AAA TAT TAC GCT CCA TGG TCT GGT CAC TGT AAA AAA TTA	1608		
	Val Leu Val Lys Tyr Tyr Ala Pro Trp Cys Gly His Cys Lys Lys Leu			
	400	405	410	415
50	GCT CCA GTC TTT GAA GAA TTA GGT GGT GTT TAT GAA TCA GTT GCT CCA	1656		
	Ala Pro Val Phe Glu Glu Leu Ala Val Tyr Glu Ser Val Ala Pro			
	415	420	425	430
55	GGT AAA GTC TTA TTA GCT GAT TTA GAT CAT ACT GAA AAT GAT GTC ACC	1704		
	Gly Lys Val Leu Leu Ala Asp Leu Asp His Thr Glu Asn Asp Val Thr			
	435	440	445	450
60	GCT GTT CAC ATT GAA CGT TAC CCA ACT ATC GTC TTA TAC CCA GCC GAT	1752		
	Gly Val His Ile Glu Gly Tyr Pro Thr Ile Val Leu Tyr Pro Ala Asp			
	455	460	465	470

GGT TCA GAA CCA GTT GTT TAG GAA CGT AAC AGA TCT TTT GAA TCT TTC 1860  
 Gly Ser Glu Pro Val Val Tyr Glu Gly Asn Arg Ser Phe Glu Ser Phe  
 8 469 470 475

TCC GAT TTC ATT AAA GAA AAA GGT TCA TCA GCT GTT GAT GCT AAT GCA 1866  
 Ser Asp Phe Ile Lys Glu Lys Gly Ser Ser Gly Val Asp Ala Asn Ala  
 16 480 483 490

TTA AAA GAA CCT TAC GCA GAA GAA GGT ACT GAA GCT GCT GCA GTT GAT 1896  
 Leu Lys Glu Pro Tyr Pro Glu Glu Gly Thr Glu Gly Ala Pro Val Asp  
 18 495 500 505 510

GCA GAA TCA GTT GCT GAT GCT GAA AAA GAA GAT GAT TCT GCT GCT GAT 1894  
 Pro Glu Ser Val Glu Asp Ala Glu Lys Glu Asp Asp Ser Ala Ala Asp  
 20 515 520 525

GTT CGT GAT GAA TTA TAAACAACTA GAATTAAATTAA TAAATTGATT AAATAAGCTT 1999  
 Val Arg Asp Glu Leu  
 22 530 531

CTAAAAATTA AATTTAAAAAT AATAAARAAA A 2030

SEQ ID No: 2

Sequence length: 4

20 Sequence type: amino acid

Topology: linear

Molecule type: peptide

22 Sequence description:  
 Lys Asp Glu Leu

SEQ ID No: 3

Sequence length: 4

24 Sequence type: amino acid

Topology: linear

Molecule type: peptide

26 Sequence description:  
 His Asp Glu Leu

SEQ ID No: 4

Sequence length: 4

28 Sequence type: amino acid

Topology: linear

Molecule type: peptide

30 Sequence description:  
 His Asp Glu Leu

5 Cys Gly His Cys  
SEQ ID No: 5  
Sequence length: 7  
Sequence type: amino acid  
Topology: linear  
Molecule type: peptide  
Sequence description:  
Pro Trp Cys Gly His Cys Lys  
S  
10 SEQ ID No: 6  
Sequence length: 7  
Sequence type: amino acid  
Topology: linear  
Molecule type: peptide  
Sequence description:  
Tyr Ala Pro Trp Cys Gly His  
S  
15 SEQ ID No: 7  
Sequence length: 29  
Sequence type: nucleic acid  
Topology: linear  
Molecule type:  
Sequence description:  
CCGGAATTCC CWTGGTCTYCC WCAYTGYAA  
SEQ ID No: 8  
Sequence length: 28  
Sequence type: nucleic acid  
Topology: linear  
Molecule type:  
Sequence description:  
CGCGGATCCT GWCCRCACCA WGGDGCR  
SEQ ID No: 9  
Sequence length: 4  
Sequence type: amino acid  
Topology: linear  
Molecule type: peptide  
Sequence description:

Arg Asp Glu Leu

8 SEQ ID No: 10

Sequence length: 40

Sequence type: nucleic acid

Topology: linear

16 Molecule type: chemical synthetic DNA

Sequence description:

ATAAGAATGC GGCGGCAAAA TGAAGTTAAC TAATTTCAA

40

18 SEQ ID No: 11

Sequence length: 38

Sequence type: nucleic acid

20 Topology: linear

Molecule type: chemical synthetic DNA

Sequence description:

ATAAGAATGC GCGGGCTTAT ATTTCATCAC GAACATCA

38

22

30

38

46

56

66

SEQUENCE LISTING

8

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Suntory Limited
- (B) STREET: 1-40, Dojimahama 2-chome, Kita-ku, Osaka-shi
- (C) CITY: Osaka
- (D) COUNTRY: Japan
- (E) POSTAL CODE (ZIP): Osaka 530

(ii) TITLE OF INVENTION: Protein disulfide isomerase gene derived  
from strain of methylotrophic yeast

20

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.26 (EPO)

28

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: EP 97306671.1

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: JP 8-234287.
- (B) FILING DATE: 04-SEP-1996

40

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2030 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

48

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: *Candida boidinii*

(B) STRAIN: S2

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

10	AGACGCCCTT GCGCTCCTTC ATTATTCATC CACTCTCTCC TCGAACGTTG TGAAACAATTTC	60
	CACTCACTTG CCTTGGTTTA CCATCTACTCA ATCGTTTCA TTATCTCCGT TATCATTCGA	120
	CCATTTCTTC ACTTTTTCAT ATCTGCTAATC AAATGCTCA AGCAACGATA ATCTTCAGG	180
	AGATTCGCTC TTCTTTGATT CAATTGATCC TTCTAGAC AGATCTCTGA GAGCTTAAATA	240
15	CTTACATAGA TATATATATA TATATATGAA ATTACTTIT CCTCAATTACT CAATGATTC	300
	CKTTTATATAC ATTCTTCTGTA TATATATTTGA CTTAAATAT ATTACATAT AGACATAAACA	360
	TTTAAAG ATG ARG TTA ATT AAT TTC AAA GTT ATT GCC ACA ATT CTT GCT	420
	Met Lys Leu Thr Asn Phe Lys Val Ile Ala Thr Ile Leu Ala	
	3                      5                          10	
20	TGT TTA ACA GTT ATT AGA GCT GAT GAT GCT GCT CCC ATT CCA TCT CCA	486
	Cys Leu Thr Val Val Arg Ala Asp Asp Gly Gly Ala Ile Ala Ser Pro	
	15                      20                      25                  30	
	GAT TCC GCT GTT GTT AAA TTA ACT GCT GAT TCA TTC GAA TCA TTC ATG	504
25	Asp Ser Ala Val Val Lys Leu Thr Ala Asp Ser Phe Glu Ser Phe Met	
	35                      40                      45	
	AAA GAA ATT CCA TTA GTC TTA GCT GAA TTT TTT GCT CCT TGG TGT GGT	522
	Lys Glu Asn Pro Leu Val Leu Ala Glu Phe Phe Ala Pro Trp Cys Glu	
30	50                      55                      60	
	CCT TGT AGA AGA TTC GCT CCT GAA TTT CAA GTC CCT CCT GAT AAA TTA	600
	Ris Cys Lys Arg Leu Glu Pro Glu Phe Glu Val Ala Ala Asp Lys Leu	
	65                      70                      75	
35	CTT GAA AGA GAT ATT AGA TTA GCT CAA ATT GAT TGT ACC GAA GAA AAA	646
	Val Glu Asp Ile Arg Leu Ala Glu Ile Asp Cys Thr Glu Glu Lys	
	80                      85                      90	
	GAT TTA TGT TCT TAT GGT ATT AAA GCT TAC CCA ACT TTA AAA GTC	666
	Ser Leu Cys Ser Ser Tyr Glu Ile Lys Glu Tyr Pro Thr Leu Lys Val	
40	95                      100                    105                  110	
	TTT AGA GGT TAC GAA ATT GAA CCT TCT GAT TAT GCT GGT CAA AGA ACT	744
	Phe Arg Glu Tyr Glu Asn Glu Pro Ser Asp Tyr Ala Glu Gln Arg Thr	
	115                    120                    125	
45	TCA GAT TCA ATC ATT TCT TAT ATG GTT AAA CAA TCA ACC CCA CCT GTC	792
	Ser Asp Ser Ile Ile Ser Tyr Met Val Lys Glu Ser Thr Pro Pro Val	
	130                    135                    140	
	TCC ATC CCT GAT GAT GTC TCA GAT ATC GAA GAT ACA ATT AAA GAA TCA	840
50	Ser Ile Val Arg Ser Leu Ser Asp Ile Glu Asp Thr Ile Lys Glu Ser	
	145                    150                    155	
	AAT GAT GGT GCT TTT ATT CAA GTC TTA CCA AAA GGT TCT AAA TCT GTT	888
	Asn Asp Pro Val Phe Val Glu Val Pro Lys Glu Ser Lys Ser Val	
	160                    165                    170	

55

	GAA	CDC	GCT	ABC	TCA	ACT	TTG	TTT	CAN	AAC	GCT	AAA	GCT	TTA	RGA	GAT	936
8	Glu	Ala	Gly	Asn	Ser	Thr	Phe	Phe	Glu	Ile	Ala	Asn	Gly	Leu	Arg	Asp	
	178				180				185				190				
	AAC	TAC	TCT	TTT	ATT	TCA	ACA	ACA	AGT	ACT	GAA	TTG	TCT	TCA	AAA	TAC	984
	Asn	Tyr	Ser	Phe	Ile	Ser	Thr	Thr	Ser	Thr	Glu	Phe	Ser	Ser	Lys	Tyr	
10							195				200			205			
	TTC	ASA	GCT	ATT	AAA	AAA	TCA	GAT	ACT	CCA	TCT	TAT	ATT	CTC	TTT	AGA	1032
	Leu	Lys	Gly	Ile	Lys	Lys	Ser	Asp	Thr	Pro	Ser	Tyr	Ile	Leu	Phe	Arg	
					210				215			220					
15	CCA	ATA	GAA	CAA	TTC	TCT	GAT	GCT	TCA	ATC	TAT	AAA	TTT	GAT	GAA	ATT	1080
	Pro	Asn	Glu	Glu	Leu	Ser	Asp	Ala	Ser	Ile	Tyr	Lys	Phe	Asp	Glu	Ile	
					225				230			235					
	GAT	GAT	ACT	CAT	TTG	ATC	GAA	TTC	TTA	AGC	GTT	GAA	TCA	AAA	GCT	TTA	1128
	Asp	Asp	Thr	His	Ile	Ile	Glu	Phe	Leu	Asn	Val	Glu	Ser	Lys	Pro	Leu	
20					240				245			250					
	TTC	GCT	GAA	ATG	GAT	GCT	TCT	TTC	CAA	TCT	ATG	ATG	AAA	ATG	AAA	1176	
	Phe	Gly	Glu	Met	Asp	Gly	Ser	Ser	Phe	Gln	Ser	Tyr	Met	Glu	Met	Lys	
					255				260			265			270		
25	TTA	CCA	GTT	GCT	TAT	TAT	TTC	TAT	AGT	GAA	ATC	TCT	GAA	AAA	GAT	GCC	1224
	Leu	Pro	Val	Ala	Tyr	Tyr	Phe	Tyr	Asn	Glu	Ile	Ser	Glu	Lys	Asp	Ala	
					275				280			285					
30	GTC	TCT	GAT	GCG	ATC	ACT	AAA	TTG	GCT	AAA	ACT	CAT	AGA	GCT	AAA	GTT	1272
	Val	Ser	Asp	Ala	Ile	Ser	Lys	Ile	Ala	Lys	Thr	His	Arg	Gly	Lys	Val	
					290				295			300					
	AAT	TTC	GCT	TTA	GAC	GCT	TOT	AAA	TAT	GCT	TTA	CAC	GCT	AGG	ATT		1320
	Asn	Phe	Val	Gly	Leu	Asp	Ala	Ser	Lys	Tyr	Gly	Leu	His	Ala	Lys	Asn	
35					305				310			315					
	ATT	AAC	ATG	AGG	GAA	TTC	CCT	TTC	GCT	ATT	CAC	GAT	TTA	CCA		1368	
	Ile	Asn	Met	Lys	Glu	Glu	Phe	Pro	Ile	Phe	Ala	Ile	His	Asp	Leu	Ala	
					320				325			330					
40	ACT	GAA	TTA	AAA	TAC	GCT	ATC	TCC	CAA	GAT	AAA	CCA	TTA	CAT	ATT	AAA	1416
	Thr	Glu	Leu	Lys	Tyr	Gly	Ile	Ser	Gln	Asp	Lys	Pro	Leu	Asp	Asn	Lys	
					335				340			345			350		
	TTA	ATT	CCA	AAA	TTC	GTT	GAA	CAT	TTC	GTT	GCT	GCT	TTA	GAG	CCA		1464
	Leu	Ile	Pro	Iys	Phe	Val	Glu	Asp	Phe	Val	Glu	Ala	Gly	Lys	Leu	Glu	
45					355				360			365					
	ATC	ATT	AAA	TCR	GAA	CCA	ATC	CCA	GAA	ACT	CAA	GAT	TCT	CCT	TAC		1512
	Ile	Ile	Lys	Ser	Glu	Pro	Ile	Pro	Ile	Thr	Gln	Asp	Ser	Pro	Val	Tyr	
					370				375			380					
50	CAT	TTC	GTC	GCT	AAA	GAA	CAT	GAT	AAA	ATT	ATT	AOX	TCT	CAT	AAA	GAT	1560
	Mis	Leu	Vai	Gly	Lys	Glu	Mis	Asp	Lys	Ile	Ile	Ser	Thr	Asp	Lys	Asp	
					385				390			395					
55	GTC	TTA	GTT	AAA	TAT	TAC	GCT	GGG	TGG	TCT	GAT	CAC	TCT	AAA	AAA	TTA	1608
	Val	Leu	Vai	Lys	Tyr	Tyr	Ala	Pro	Trp	Cys	Gly	Mis	Cys	Lys	Lys	Leu	
					400				405			410					

5 GCT CCA GTC TTT GAA GAA TTA GCT GCT GTT TAT GAA TCA GTT GCT CCA 1656  
 Ala Pro Val Phe Glu Glu Leu Ala Ala Val Tyr Glu Ser Val Ala Pro  
 415 420 425 430  
 GGT AAA GTC TTA TTA OCT GAT TTA GAT GAT ACT GAA AAT GAT GTC ACC 1704  
 Gly Lys Val Leu Leu Ala Asp Leu Asp His Thr Glu Asn Asp Val Thr  
 435 440 445  
 10 GGT GTT CAC ATT GAA GGT TAC CCA ACT ATC GTC TTA TAC CCA CCC GAT 1752  
 Gly Val His Ile Glu Gly Tyr Pro Thr Ile Val Leu Tyr Pro Ala Asp  
 450 455 460  
 GGT TCA GAA CCA GTT GTT TAC GAA GGT AAC AGA TCT TTT GAA TCT TTC 1800  
 Gly Ser Glu Pro Val Val Tyr Glu Gly Asn Arg Ser Phe Glu Ser Phe  
 465 470 475  
 15 TCC GAT TTC ATT AAA GAA GAA GGT TCA TCA GGT GTT CAT GCT GGT ATT GCA 1848  
 Ser Asp Phe Ile Lys Glu Lys Gly Ser Ser Gly Val Asp Ala Asn Ala  
 480 485 490  
 TTG AAA GAA CCT TAC CCA GAA GAA GGT ACT GAA GGT CCT CCT CCA GTT GAT 1896  
 Leu Lys Glu Pro Tyr Pro Glu Glu Gly Thr Glu Gly Ala Pro Val Asp  
 495 500 505 510  
 20 CCA GAA TCA GTT GGT GAT GCT GAA GAA GAT GAT GAT TCT GCT GGT GAT 1944  
 Pro Glu Ser Val Gly Asp Ala Glu Asp Asp Ser Ala Ala Asp  
 515 520 525  
 GTT CCT GAT GAA TTA TAAACAGTA GAAATTAATTA TAACTTCATT AAATAGCTTT 1999  
 25 Val Arg Asp Glu Leu  
 530 531  
 CTAAAGATTA TATTAAATTA AATAAAAGAA A 2030

## (2) INFORMATION FOR SEQ ID NO: 2:

- 30 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 4 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:  
 Lys Asp Glu Leu

## (2) INFORMATION FOR SEQ ID NO: 3:

- 40 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 4 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

8 (ii) MOLECULE TYPE: peptide

16 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:  
His Asp Glu Leu

18 (2) INFORMATION FOR SEQ ID NO: 4:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

22 (ii) MOLECULE TYPE: peptide

24 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:  
Cys Gly His Cys

28 (2) INFORMATION FOR SEQ ID NO: 5:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

38 (ii) MOLECULE TYPE: peptide

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:  
Pro Trp Cys Gly His Cys Lys

5

46 (2) INFORMATION FOR SEQ ID NO: 6:

48 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

56 (ii) MOLECULE TYPE: peptide

68

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

8      Tyr Ala Pro Trp Cys Gly His  
       5

(2) INFORMATION FOR SEQ ID NO: 7:

- 10     (i) SEQUENCE CHARACTERISTICS:  
       (A) LENGTH: 29 base pairs  
       (B) TYPE: nucleic acid  
 15     (C) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

20     CGGGGATTCG CWTGGTGTGCG WCAATGAA

29

(2) INFORMATION FOR SEQ ID NO: 8:

- 25     (i) SEQUENCE CHARACTERISTICS:  
       (A) LENGTH: 28 base pairs  
       (B) TYPE: nucleic acid  
 30     (C) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

35     CGGGGATCC CWCCTCACCA WGGGGCRT

28

(2) INFORMATION FOR SEQ ID NO: 9:

- 40     (i) SEQUENCE CHARACTERISTICS:  
       (A) LENGTH: 4 amino acids  
       (B) TYPE: amino acid  
       (D) TOPOLOGY: linear

45     (ii) MOLECULE TYPE: peptide

50     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:  
       Arg Asp Glu Leu

55

(2) INFORMATION FOR SEQ ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(D) TOPOLOGY: linear

(II) MOLECULE TYPE: Other nucleic acid; chemical synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ATAGAATGCGCCCGTAAAC TGAGGTAAAC TATTTCGAA

40

(2) INFORMATION FOR SEQ ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 base pairs

(B) TYPE: nucleic acid

(D) TOPOLOGY: linear

(II) MOLECULE TYPE: Other nucleic acid; chemical synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATAGAATGCGCCCGTTAT ATTCATCAC GACATCA

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**Claims**

1. A protein derived from a strain of methylotrophic yeast which has a protein disulfide isomerase activity, having the amino acid sequence as set forth in SEQ ID No. 1, or protein in which said amino acid sequence has been modified by deletion or addition of one or a few amino acids, or substitution with other amino acid(s) and which has a protein disulfide isomerase activity.
2. A protein derived from a strain of methylotrophic yeast, which has the conserved region of the active center for protein disulfide isomerase comprising Cys-Gly-His-Cys (SEQ ID No. 4) and an endoplasmic reticulum retention signal sequence comprising Arg-Asp-Glu-Leu (SEQ ID No. 8), and which has a protein disulfide isomerase activity.
3. A gene encoding a protein according to claim 1 or 2.
4. A gene according to claim 3 represented by the nucleotide sequence as set forth in SEQ ID No. 1.
5. A vector comprising a gene according to claim 3 or 4.

6. A transformant obtained by transforming a host with a vector according to claim 5.
7. A transformant according to claim 6 wherein the host is a strain of methylotrophic yeast;
8. A transformant according to claim 7 wherein the strain of methylotrophic yeast is Candida boidinii;
9. A method for producing a protein having a protein disulfide isomerase activity, which method comprises culturing a transformant according to any one of claims 6 to 8, and then recovering the protein from the culture.
10. A method for producing a peptide or a protein encoded by a heterologous structural gene, which method comprises culturing a transformant cotransformed with a vector according to claim 5 and a vector having a heterologous structural gene and then recovering an expression product of the heterologous structural gene, which is the peptide or the protein, from the culture.
11. A method for producing a peptide or a protein encoded by a heterologous structural gene, which method comprises culturing a transformant cotransformed with a vector which contains the recombinant gene of the protein disulfide isomerase of a methylotrophic yeast and a vector having a heterologous structural gene and then recovering an expression product of the heterologous structural gene, which is the peptide or the protein, from the culture.

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Fig. 1

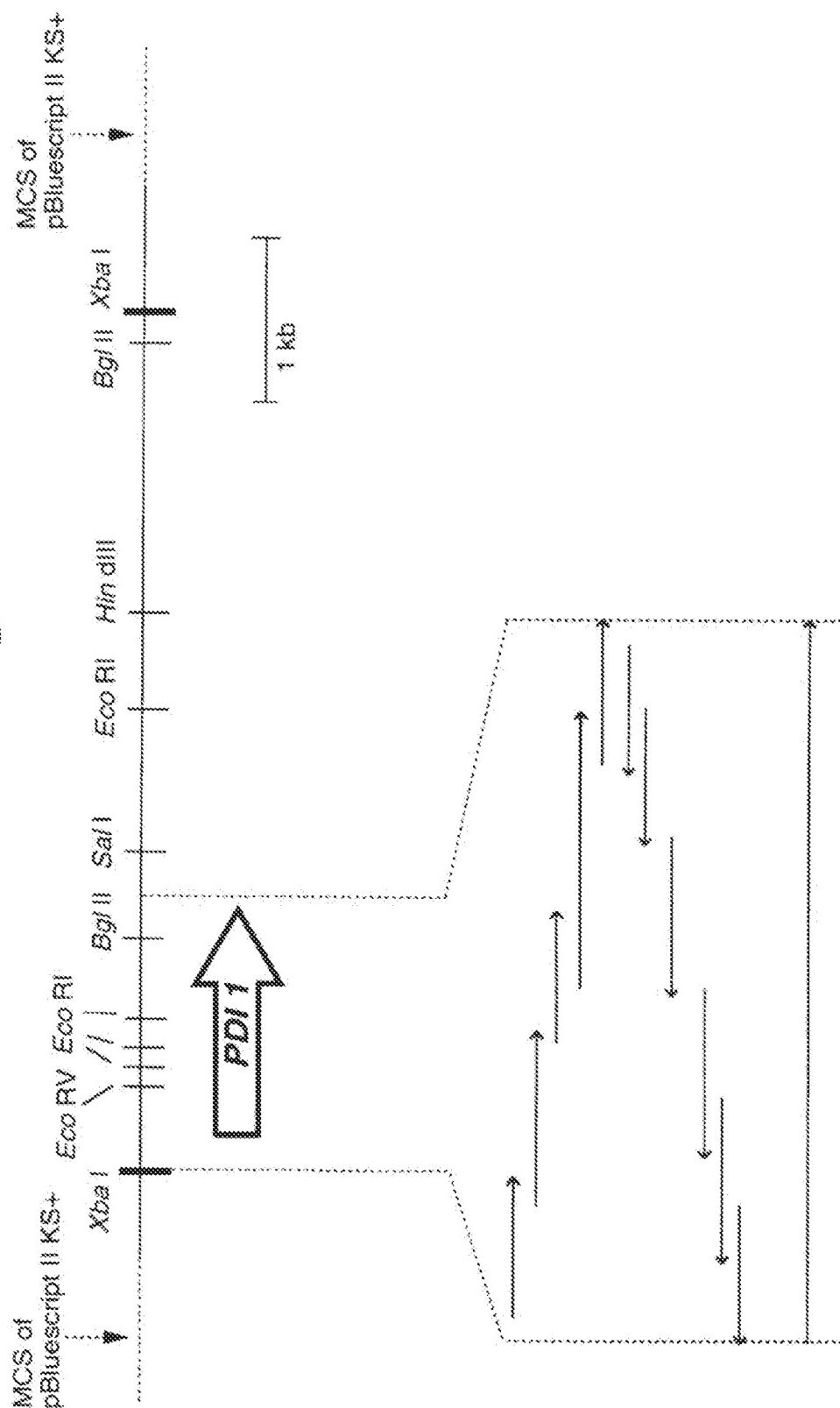


Fig. 2

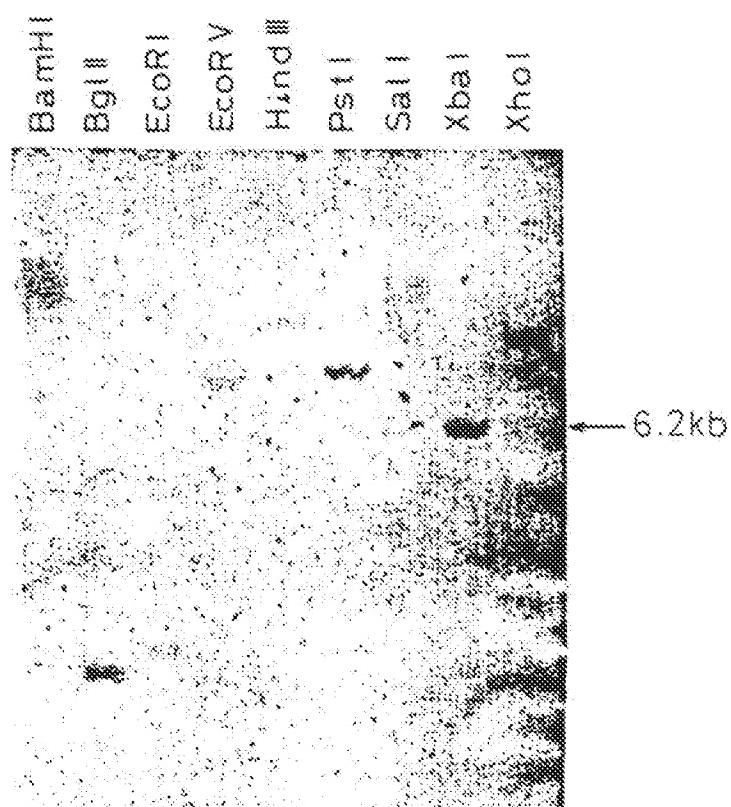


Fig. 3

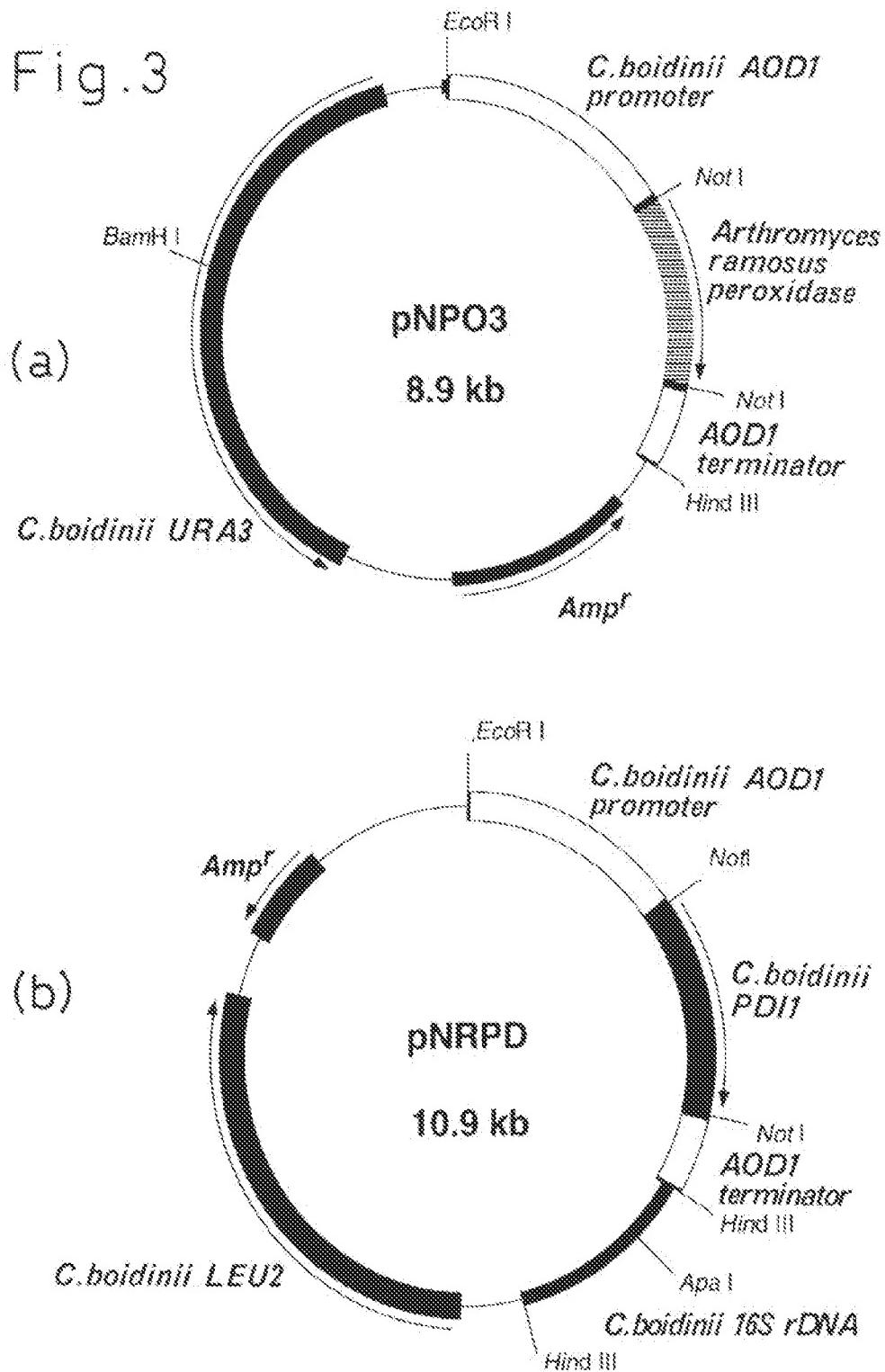


Fig. 4

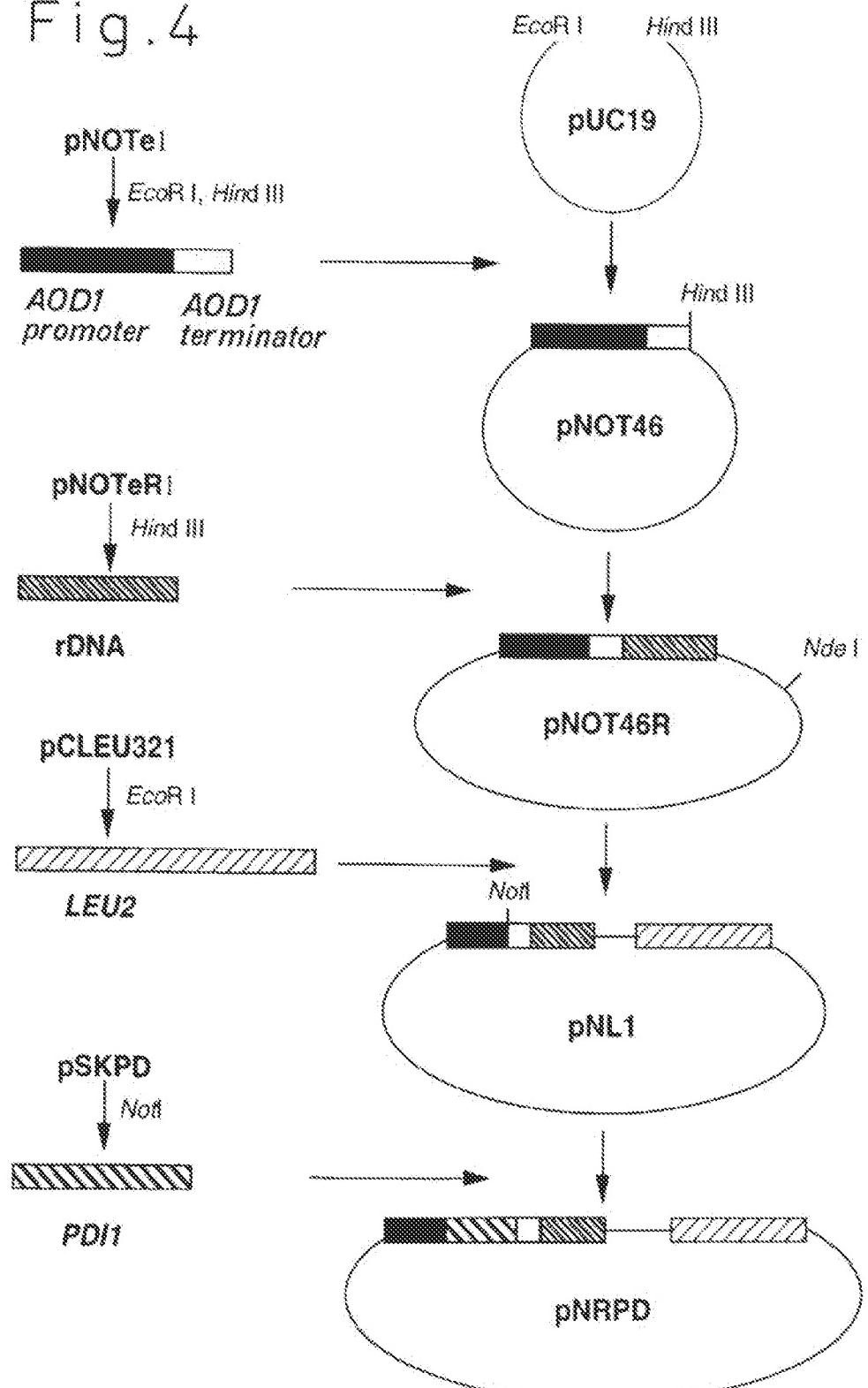


Fig. 5

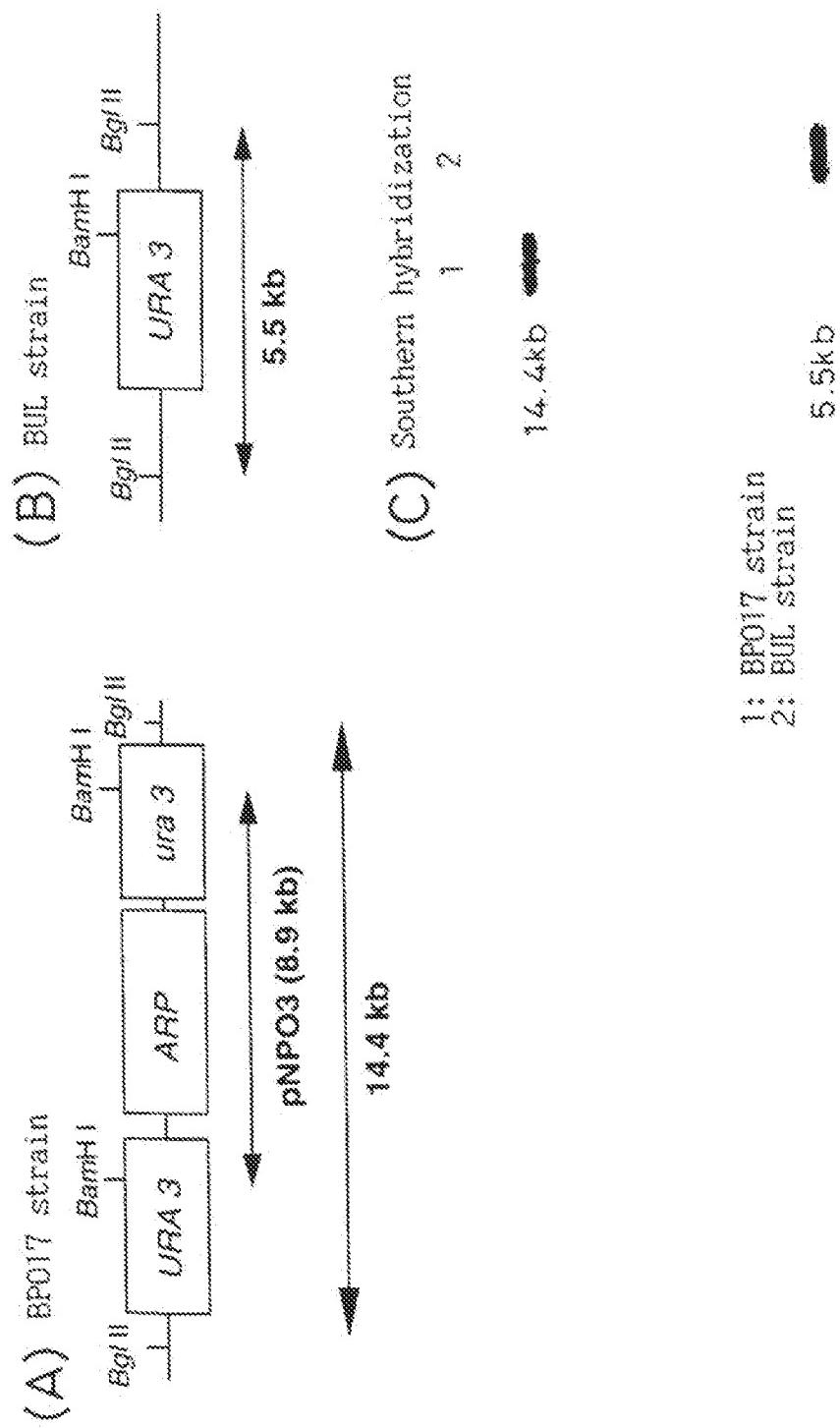


Fig. 6

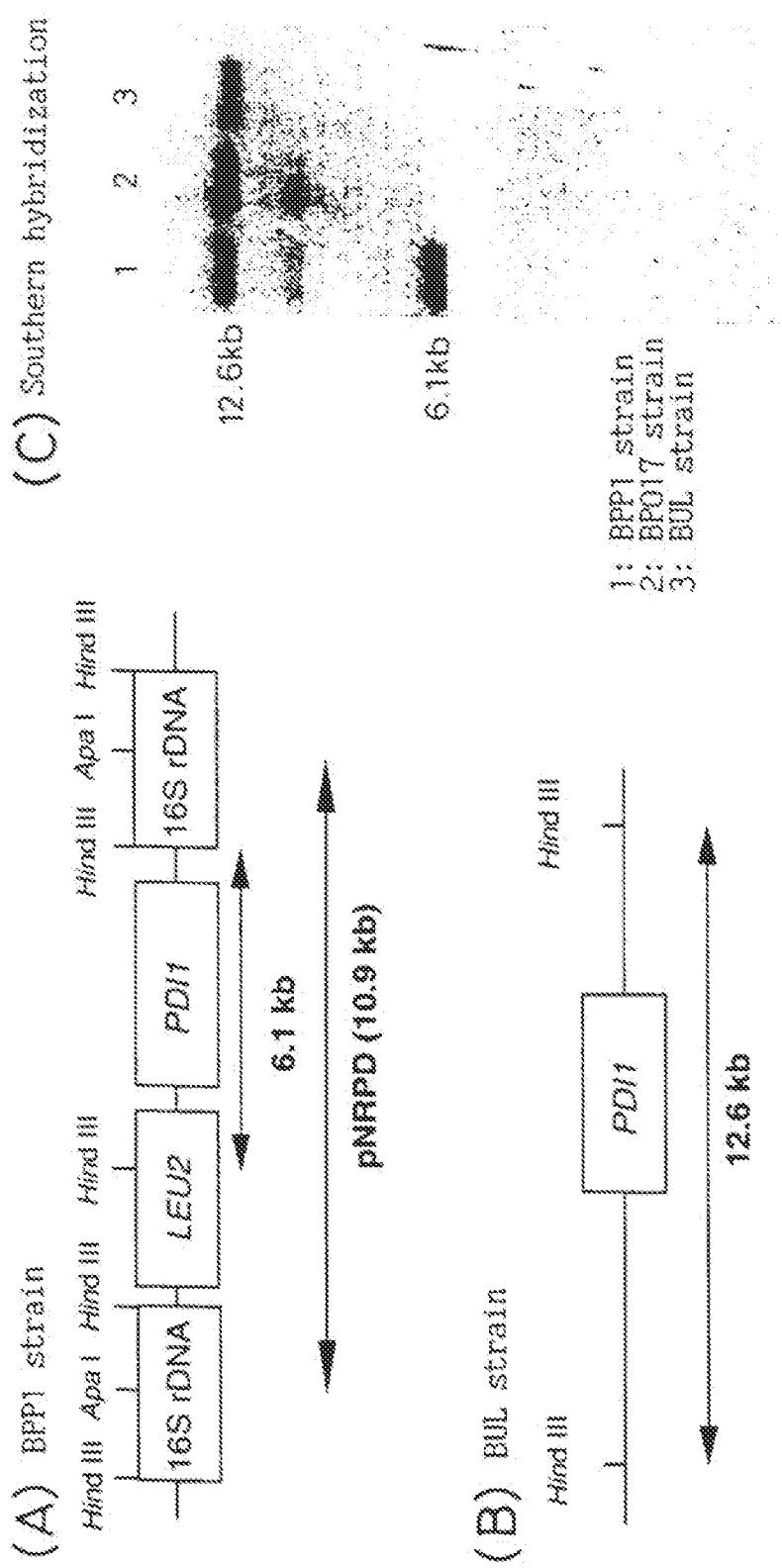
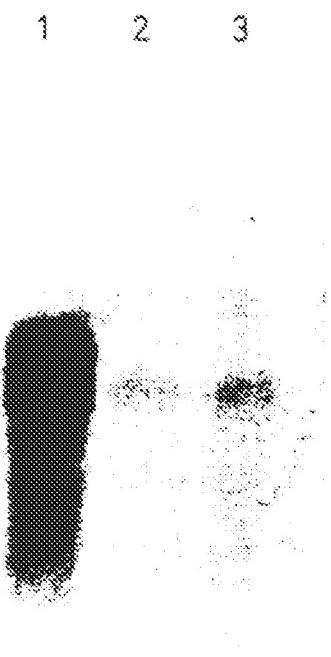


Fig.7



PDI

- 1: BPP1 strain
- 2: BPO17 strain
- 3: BUL strain

Fig. 8

